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SHORT COMMUNICATION

IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity *in vivo*

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Th17 cells have been recognized as the central effectors in organ-related autoimmune diseases. IL-6 is a key factor that reciprocally regulates Th17 and Foxp3$^+$ Treg differentiation by inhibition of TGF-β induced Foxp3 and induction of RORγt, a Th17 lineage-specific transcription factor. Recently IL-21 has been suggested to induce RORγt and Th17 development in the absence of IL-6. However, the relevance of IL-21 for Th17-dependent inflammatory responses *in vivo* remains unclear. In this study, we demonstrate that differentiation of IL-17-producing CD4 T cells, their recruitment to inflamed organs, and the development of autoimmune disease was not affected in *il21R*$^{-/-}$ and *il21*$^{-/-}$ mice in models of myelin oligodendrocyte glycoprotein-induced autoimmune encephalitis and autoimmune myocarditis. IL-6 induced Th17 differentiation independent of and much more potently than IL-21 *in vitro*. These data suggest that IL-6 is sufficient to drive Th17 development and associated autoimmunity *in vivo* in the absence of IL-21 or IL-21R.

Key words: Autoimmunity · EAE · IL-21 · Myocarditis · Th17

See accompanying Commentary by Holmdahl

Supporting Information available online

Introduction

CD4$^+$ T helper (Th) cells orchestrate immune responses by differentiating into discrete subsets characterized by distinct cytokine secretion patterns. Th2 cells shape effector responses during allergy and helminth infections by secretion of a panel of cytokines including IL-4, IL-5, IL-10, and IL-13. Th1 cells mediate control of bacterial, protozoan, and fungal infection by production of IFN-γ, a key cytokine for the activation of classical macrophages and production of nitric oxide and reactive oxygen intermediates. Th1 cells have also been implicated in the inflammatory pathology associated with organ-related autoimmune diseases, a concept challenged by reports showing normal or even exacerbated autoimmune inflammation in IL-12-deficient (p35$^{-/-}$) and IFN-γ$^{-/-}$ mice [1–6]. The finding that IL-23$^{-/-}$ mice did not develop EAE [7] due to reduced IL-17 production sparked interest in a novel T cell population termed Th17 cells, which secrete a unique profile of cytokines including IL-17, IL-17F, and IL-22 [8, 9]. Besides EAE, Th17 cells have been suggested to mediate a number of inflammatory diseases including experimental arthritis [10], myocarditis [11] and colitis [12]. Similar to reciprocal development of Th1 and Th2 cells induced by activation of the transcription factors Tbet and GATA3, respectively, recent studies demonstrated reciprocal development of Th17 cells and regulatory T cells (Treg) [13] by induction of lineage-specific transcription factors through cytokines. TGF-β-guided Foxp3 expression supports Treg development, while TGF-β- together with IL-6 directs Th17 differentiation by induction of RORγt [14]. IL-23, the stumbling block of the Th17 story [7, 15], has been proposed to expand and maintain previously differentiated Th17 cells [13, 16]. By contrast, IL-27 [17, 18], IL-4, IFN-γ [16], IL-2 [19], and retinoic

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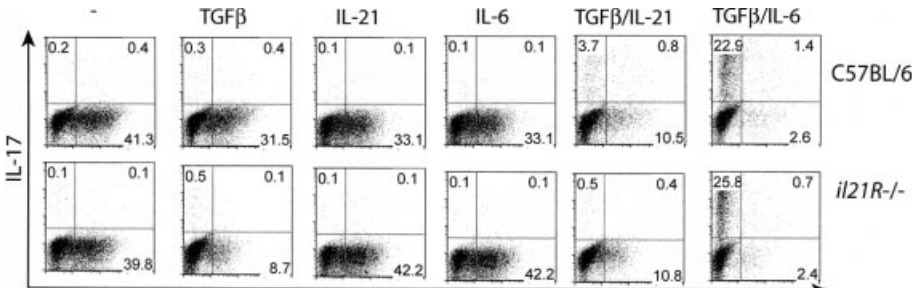

Figure 1. IL-21 mediated development of Th17 cells is abrogated in the absence of IL-21R. $CD4^+$ T cells were purified from naïve wild-type and $il21R^{-/-}$ mice and cultured on plates bound with anti-CD3 together with anti-CD28 in the presence of cytokines where indicated; TGF-β1 (5 ng/mL), IL-6 (20 ng/mL), IL-21 (20 ng/mL). After 5 days of culture cells were restimulated with PMA and ionomycin for 5 h with Brefeldin A for the last 3 h. After intracellular staining of IL-17 and IFN-γ, cells were analyzed by flow cytometry.

acid [20] negatively regulate Th17 development. IL-2 and retinoic acid have been shown to cross-regulate Treg and Th17 development by promoting Foxp3 and inhibiting RORγt, opposite to IL-6 [19, 20].

Three publications recently described an important role of IL-21 in the generation of Th17 cells [21–23]. Elegant *in vitro* studies showed that IL-21, along with TGF-β, inhibited Treg and induced Th17 differentiation by activation of RORγt and STAT3 independent of IL-6. An autocrine mechanism for IL-21-mediated Th17 differentiation has been proposed because of predominant expression of IL-21 in Th17 cells as compared to Th1, Th2, and Treg cells. Confirming these data, we here show that IL-21 can drive Th17 differentiation *in vitro*, although much less potent than IL-6. However, *in vivo* IL-21 or IL-21R was dispensable for development of Th17 cells and associated autoimmune diseases in models of EAE and experimental autoimmune myocarditis (EAM).

Results and discussion

IL-21 promotes development of Th17 cells *in vitro*

To assess the role of IL-21 in differentiation of Th17 cells *in vitro*, purified naïve $CD4^+$ T cells from wild-type and $il21R^{-/-}$ mice were stimulated with plate-bound anti-CD3, anti-CD28, and TGF-β in combination with IL-6 or IL-21. Consistent with published results, TGF-β/IL-21 promoted Th17 differentiation, which was abrogated in CD4 T cells from $il21R^{-/-}$ mice (Fig. 1). Notably, IL-6/TGF-β stimulation induced a much higher frequency of IL-17-producing cells compared to IL-21/TGF-β, and IL-6-induced Th17 differentiation was unaffected in the absence of IL-21R on CD4 T cells (Fig. 1). This may suggest that the presence of high levels of IL-6 during autoimmune inflammatory responses *in vivo* may be sufficient to compensate for the absence of IL-21.

In fact, we recently reported that $il21R^{-/-}$mice develop unimpaired autoimmune myocarditis [24], an IL-23- and IL-17-dependent heart inflammatory disease in genetically susceptible BALB/c mice [11]. Extending these data, we found that frequencies of IFN-γ– and IL-17-producing CD4 T cells in the inflamed heart were comparable in $il21R^{-/-}$ and BALB/c control mice immunized with a peptide from α-myosin heavy chain ($myhc_{614–629}$) (Supporting Information Fig. 1).

Development of Th17 cells and EAE is unaffected in $il21R^{-/-}$ and $il21^{-/-}$ mice

To address the possibilities that IL-21 is important for CNS rather than heart autoimmune inflammatory disease and Th17 cell development, or that requirement of IL-21-mediated Th17 differentiation is dependent on the genetic background (*i.e.*, BALB/c *versus* C57BL/6), we studied development of myelin oligodendrocyte glycoprotein (MOG)-induced EAE in $il21R^{-/-}$ mice (N7 C57BL/6). Mice were immunized with $MOG_{35–55}$/CFA and disease score was monitored over 4 weeks. Fig. 2A shows that onset of disease and average clinical scores were comparable in $il21R^{-/-}$ mice and C57BL/6 wild-type controls. Notably, the frequency of $il21R^{-/-}$ mice with maximum disease score 3.5 was higher compared to controls (Fig. 2B) and some $il21R^{-/-}$ had to be euthanized at the peak of EAE due to disease severity (score ≥4). $CD4^+$ T cells isolated from the inflamed CNS and the spleen showed that frequencies of IL-17- and IFN-γ-producing cells were not altered in $il21R^{-/-}$mice (Fig. 2C).

To exclude possible differences between $il21^{-/-}$ and $il21R^{-/-}$ mice, we investigated MOG-induced EAE in $il21^{-/-}$ mice. In accordance with results above, $il21^{-/-}$ mice presented with massive EAE, which appeared even exacerbated based on percentage of mice reaching maximum disease score (set at 3.5) (Fig. 3A, B). Again some knockouts had to be euthanized due to disease severity (score ≥4) according to animal protection guidelines. It should be noted that $il21R^{-/-}$ and $il21^{-/-}$mice used for these experiments were backcrossed to C57BL/6 for 7 and 3 generations, respectively, and we have used pure C57BL/6 as controls, which may impose a limitation for a definitive conclusion. Future experiments with large numbers of wild-type and knockout littermates, extensive immunohistology, and possibly the inhibition of IL-21 at different phases of EAE are required to answer whether absence of IL-21 may worsen outcome of disease as indicated (Fig. 2B, 3B).

The ratio of Th17 and $FoxP3^+$ Treg cells critically influences development of EAE, as shown in IL-6-deficient mice, which are

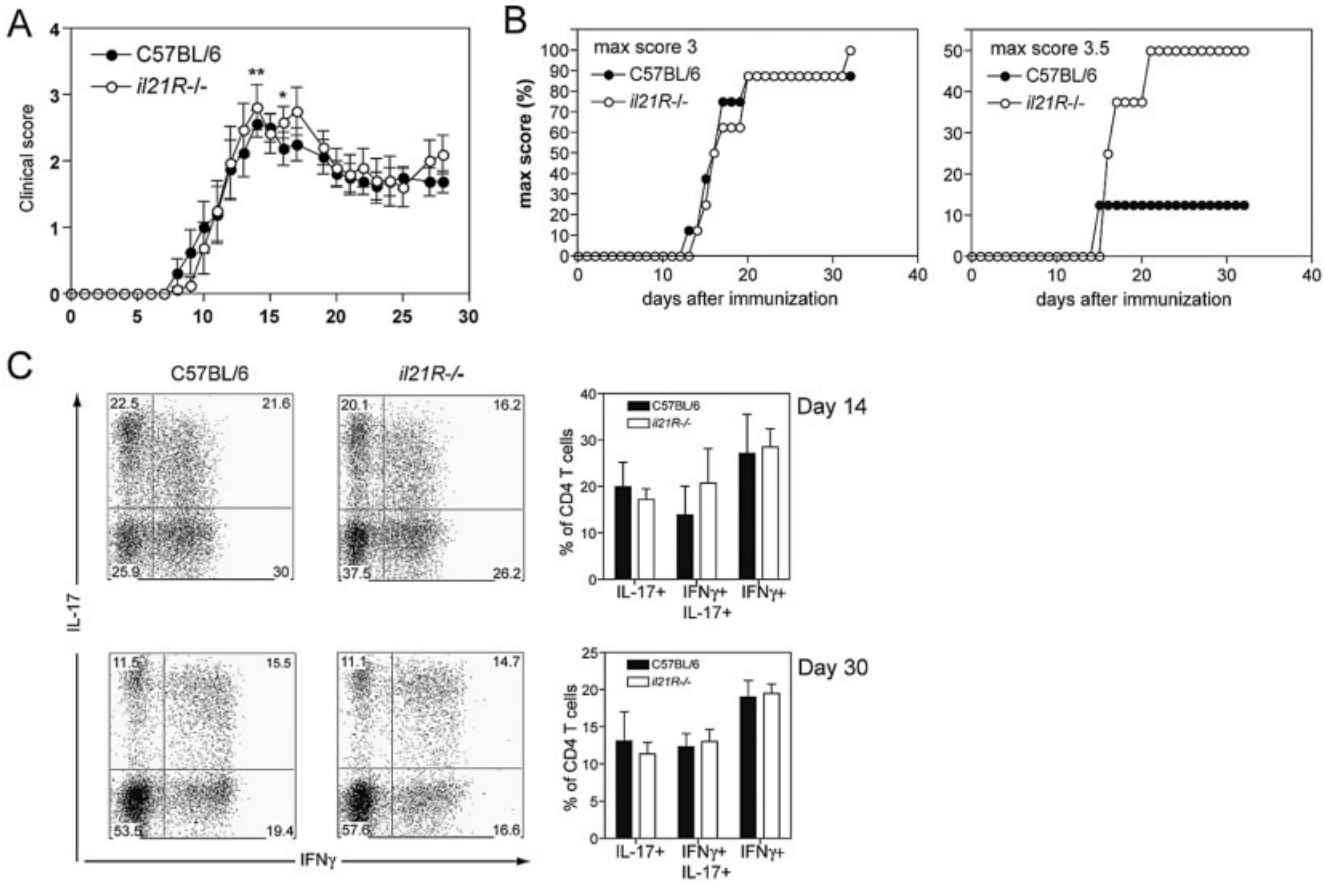

Figure 2. Normal EAE development and cytokine production by inflammatory CD4 T cells in the absence of IL-21R. Wild-type and *il21R*$^{-/-}$ mice (n=8/group) were immunized with MOG_{35-55} emulsified in CFA. (A, B) Mice were scored longitudinally for paralysis as described in *Materials and methods*. (A) Shown are averages ± SEM. Three *il21R*$^{-/-}$ mice were euthanized due to disease severity (score ≥4) at indicated days (*). (B) Prevalence of disease scores set at 3 and 3.5 (C) MNC of the CNS (spinal cord and brain) were isolated at days 14 (upper row) and 30 (lower row) after immunization and restimulated with PMA/ionomycin and Brefeldin A for 2.5 h. IL-17 and IFN-γ production by CD4$^+$ T cells was assessed by intracellular staining and flow cytometry. Dot plots of a sample representative for the group. Histograms indicate average of three to four mice per group (± SD). One representative experiment of two is shown.

protected from disease due to an expansion of Treg at the expense of effector T cells [21]. IL-21 has been shown to cross-regulate Treg and Th17 differentiation by promoting Th17 and inhibiting induction of FoxP3$^+$ Treg cells *in vitro*, similar to IL-6 [21]. We found comparable frequencies of IL-17- and IFN–γ-producing CD4 T cells in the CNS of *il21*$^{-/-}$ and C57BL/6 mice (Fig. 3C), consistent with results obtained in *il21R*$^{-/-}$ mice. Moreover, FoxP3$^+$CD4$^+$ T cell numbers in the CNS (Fig. 3D) and spleen (data not shown) were not significantly different comparing *il21R*$^{-/-}$, *il21*$^{-/-}$ and wild-type mice, although they tended to be slightly increased in the knockouts.

A broad range of activities of IL-21 on B cells, NK cells, CD8 T cells, Th1, and Th2 cells have been proposed [25], but *in vivo* mechanisms and relevance for immune responses and diseases remain poorly understood. IL-21 has been shown to suppress IFN-γ production in CD4 T cells in Th1 polarizing conditions *in vitro* [26], while *il21R*$^{-/-}$ mice mounted efficient IFN-γ (Th1) responses to infection with *L. major* [24] or in CNS and heart autoimmunity (Fig. 2, 3; and Supporting Information Fig. 1). By contrast, *il21R*$^{-/-}$ mice showed impaired Th2 responses in models of asthma and nematode infection [24], although Th2 cell differentiation occurs independent of IL-21 *in vitro* (I.S., M.K., data not shown).

Concluding remarks

Our data here demonstrate that the absence of IL-21 or IL-21R does not critically influence FoxP3$^+$ Treg and Th17 cell differentiation, organ recruitment, and associated inflammatory diseases in models of CNS and heart autoimmunity. While IL-21 can drive Th17 polarization *in vitro*, IL-6 is much more potent and seems to be sufficient for Th17-mediated autoimmunity in the absence of IL-21 *in vitro* and *in vivo*. These results are important for strategies considering IL-21 as possible target for treatment of autoimmune diseases.

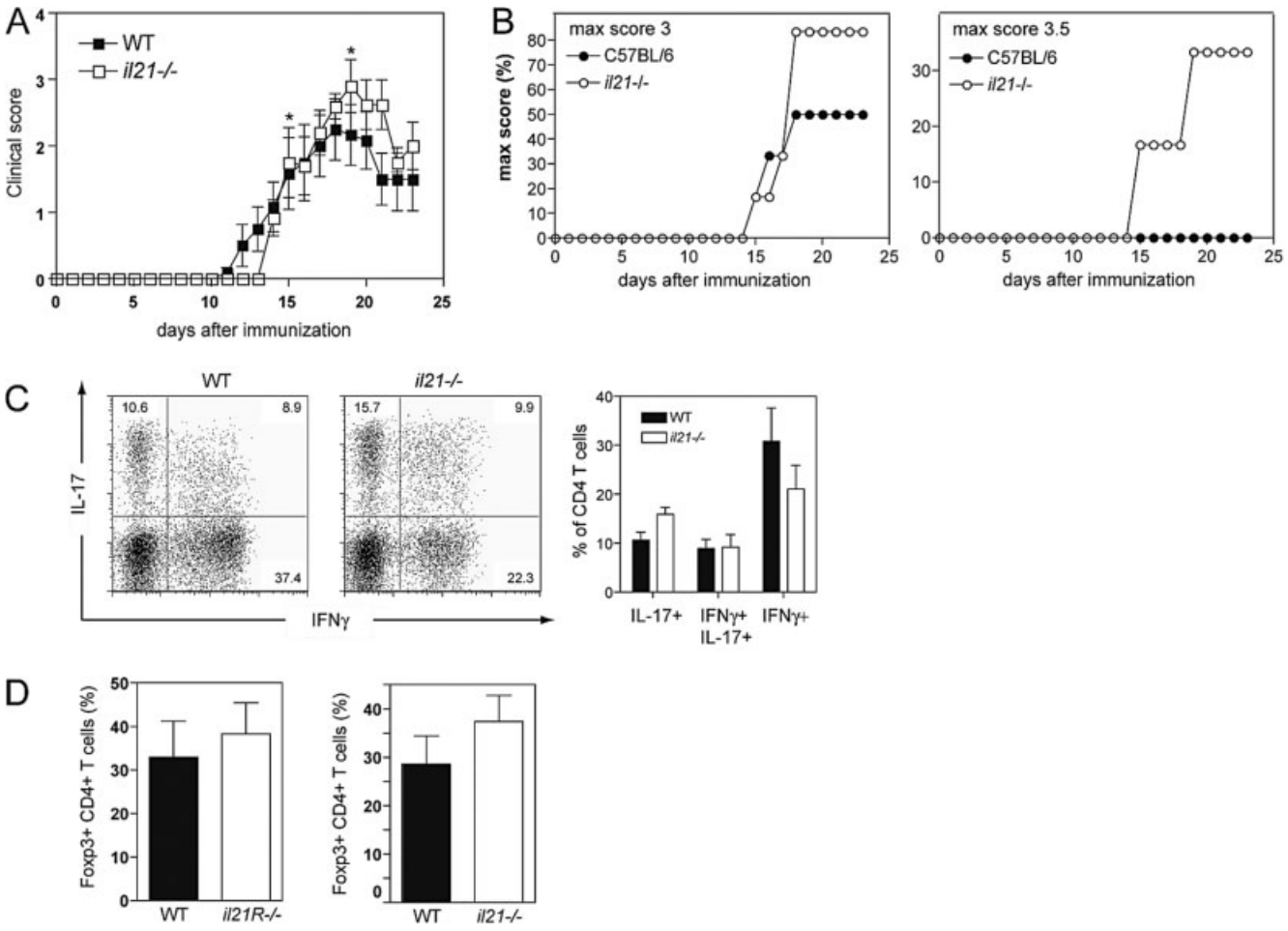

Figure 3. *il21*$^{-/-}$ mice show unaltered development of Treg, Th17 cells, and CNS inflammation. *il21*$^{-/-}$ and C5/BL/6 wild-type mice (*n*=6/group) were immunized with MOG_{35-55} emulsified in CFA. (A, B) Mice were scored for paralysis as described in *Materials and methods*. (A) Shown are averages ± SEM. Two *il21*$^{-/-}$ mice were euthanized due to disease severity (score ≥4) at indicated days (*). (B) Prevalence of disease scores set at 3 and 3.5. (C, D) MNC of the CNS (spinal cord and brain) were isolated 23 days after immunization. (C) IL-17- and IFN-γ-producing CD4 T cells were determined by intracellular staining and flow cytometry as described. Left panel shows dot plots gated on $CD4^+$ T cells of a representative mouse per group. Right panel shows a histogram with values indicating averages of four mice per group (± SD). (D) Percentage of $FoxP3^+$ $CD4^+$ T cells in CNS MNC from *il21*$^{-/-}$, *il21R*$^{-/-}$, and C57BL/6 mice determined by flow cytometry. Values show averages (± SD) of four mice per group.

Materials and methods

Mice and antibodies

Wild-type C57BL/6 and BALB/c mice were purchased from Charles River (Sulzfeld, Germany). *il21R*$^{-/-}$ mice were generated and backcrossed to C57BL/6 (N7) and BALB/c (N5) as described previously [24]. Mice were maintained in isolated ventilated cages at BioSupport (Zürich, Switzerland). *il21*$^{-/-}$ mice (B6:129S5-IL21tn1Lex) originally obtained from NIH MMRRC (F2 129/SvEvBrd x C57BL6/J) were backcrossed three generations to C57BL/6J at the Garvan Institute, Sydney. *il21*$^{-/-}$ (N3 C57BL/6J) were provided together with C57BL/6J control from the same animal facility at Garvan Institute for the experiment shown in Fig. 2. Animal experiments were approved and performed under the guidelines set by the State Veterinary Office, Zürich, Switzerland.

Labeled antibodies specific for IL-17, IFN-γ, and CD4 were purchased from eBioscience (San Diego, USA). Anti-CD3 was purified from hybridoma (145–2C11).

Induction of EAM and EAE

Myocarditis was induced essentially as described. Mice were euthanized and perfused with PBS at day 21. Hearts were removed and digested for 1 h at 37°C in 10 mg/mL collagenase. After extensive washing cells were filtered through a 70-μm and 40-mm mesh and used for intracellular staining.

EAE was induced by s.c. immunization with 100 μg/0.2 mL MOG_{35-55}, (ANAWA, Switzerland). Peptide was dissolved in PBS

and emulsified in CFA at a 1:1 ratio. Thereafter, pertussis toxin was injected i.p. (400 ng/0.2 mL PBS). Mice were monitored longitudinally and clinical disease was scored: 0, normal; 0.5, distal tail paralysis; 1, complete tail paralysis; 1.5, complete tail paralysis and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, bilateral hind limb paralysis; 3.5, bilateral hind limb paralysis and unilateral front limb paralysis; 4, bilateral hind and front limb paralysis; and 5, death.

Isolation of CNS mononuclear cells

Mice were euthanized and perfused by injecting PBS into the left heart ventricle. Brain and spinal cord were digested for 30 min at 37°C in collagenase/dispase (1 mg/mL) and DNase (10 mg/mL). After generation of a single-cell suspension, mononuclear cells (MNC) were enriched by performing a 30/70% Percoll (Amersham Bioscience) gradient. MNC were used for intracellular cytokine staining.

Restimulation and intracellular cytokine staining

Splenocytes, CNS MNC, or heart MNC were stimulated with PMA (1×10^{-7} M), ionomycin (1 μg/mL) and Brefeldin (1 μg/mL) for 3 h. Cells were stained with anti-CD4 before fixation with 2% formalin and permeabilization with 0.5% saponin, followed by incubation with cytokine-specific antibodies and flow cytometry (FACSCalibur, BD).

In vitro T cell polarization

CD4 T cells were isolated with magnetic beads (Miltenyi Biotech) from spleens of naïve mice and cultured in 96-well plates (1×10^5 cells/well) for 5 days in the presence of TGF-β (5 ng/mL), human IL-6 (20 ng/mL), or mouse IL-21 (20 ng/mL). Intracellular cytokine production was analyzed as described above.

Statistical analysis

For continuous data, significance was calculated using the unpaired Student's *t*-test. The confidential interval was 95%. Statistical evaluation was performed with the help of Prism 4 (Graphpad inc.) software.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **EAM:** experimental autoimmune myocarditis · **MNC:** mononuclear cells

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